Development of novel bacterial anti-adherence coatings for hydroxyapatite

Cukkemane, N.

2014

document version
Publisher's PDF, also known as Version of record

CHAPTER 5

Sphingosines affect the adherence of *Streptococcus gordonii* and *Streptococcus sanguinis* on hydroxyapatite.

Nivedita Cukkemane, Floris J. Bikker, Kamran Nazmi,

Henk S. Brand and Enno C.I. Veerman

Submitted

Abstract

Sphingosines exhibit bactericidal activity on planktonic cells. However, their anti-adherence activity when adsorbed on hydroxyapatite (HA) surfaces is unknown. Therefore this study evaluated the anti-adherence activity of a variety of sphingosines and their derivatives against *Streptococcus gordonii* and *Streptococcus sanguinis*. HA discs were treated with sphingosines, and subsequently incubated with *S. gordonii* or *S. sanguinis*. The number of surface-adherent bacteria was evaluated by culturing and microscopy. The anti-adherence activity of sphinganine was evaluated on 24 h old biofilms in the presence and absence of sucrose. Among the tested sphingosines, sphinganine inhibited adherence of *S. gordonii* and *S. sanguinis* by 80- and 100-fold, respectively. Growth of *S. gordonii* was inhibited 75-fold in the presence and absence of sucrose, while the growth of *S. sanguinis* was inhibited only in the absence of sucrose. These results suggest that sphinganine display potential as coating material to prevent biofilm formation on HA.

Introduction

In the oral cavity, the acquired dental pellicle provides sites for the attachment of primary colonizing streptococcal species (Rogers et al. 2001; Kolenbrander et al. 2002). Both *Streptococcus gordonii* and *Streptococcus sanguinis* are pioneers in colonizing the tooth surface (Rosan and Lamont 2000). *S. gordonii* binds to acidic proline-rich proteins and salivary amylase in the pellicle, while *S. sanguinis* binds to a complex enriched in secretory immunoglobulin A and alpha-amylase (Gibbons et al. 1991; Gong et al. 2000; Rogers et al. 2001). *S. sanguinis* has been identified as a low-pH non-mutans streptococcal species which can produce similar amounts of acid as *S. mutans* and hence may be a potential caries causing microorganism (Sansone et al. 1993; Domon-Tawaraya et al. 2013). Once adhered, *S. gordonii* co-aggregates with other bacteria such as the periopathogen *Porphyromonas gingivalis* (Haffajee and Socransky 1994). *S. gordonii* also promotes the adherence of yeast such as *Candida albicans* (Kuboniwa et al. 2006; Lamont et al. 2002; Egland et al. 2001; Jenkinson et al. 1990; O'Sullivan et al. 2000).

Dental biofilms, e.g. the dental plaque, are implicated in oral diseases, such as caries, gingivitis and periodontitis, making their control necessary (Baehni and Takeuchi 2003). The current method to control biofilms involves treatment with antimicrobial agents such as cetylpyridinium chloride, chlorhexidine digluconate, ethanol, nisin, sodium lauryl sulfate, and triclosan. However *in vitro* studies with these antimicrobial agents revealed that these treatments resulted in partial removal of biofilms (Corbin et al. 2011).

The lack of efficient biofilm control measures has sparked interest in alternative strategies aimed at inhibiting development of biofilms on surfaces, for instance by inhibiting the interaction between bacteria and the biomaterial surface (Ofek et al. 2003). Examples of such interventions are plant lectins and polyphenols which bind to carbohydrates on the surface of oral streptococci, resulting in a decreased adherence to saliva coated surfaces (Oliveira et al. 2007; Ooshima et al. 1993). Other examples are compounds which bind to the dental surface, thereby modifying its physicochemical properties. This may influence the initial adhesion of microorganisms, which is governed by non-specific physicochemical interactions. For example, phosphorylated polyethylene glycol and pyrophosphate inhibit adherence of *S. mutans* to an in vitro salivary pellicle on HA completely (Shimotoyodome et al. 2007). In a previous study we have developed HA-binding peptides and peptide conjugates which inhibit adherence of *S. mutans* (Bikker et al. 2013).

In the present study the feasibility of another class of biomolecules, the lipids, as anti-adherence coating on HA was explored. It was hypothesized that lipid films on HA may modify its physicochemical properties, thereby hampering the adherence of bacteria. In the present study sphingosines and their derivatives, which are positively charged lipids, were tested for their ability to cover HA with an anti-adherence coating. The anti-adherence property of this coating was tested against *S. gordonii* and *S. sanguinis*.

Materials and Methods

Bacterial species and growth conditions

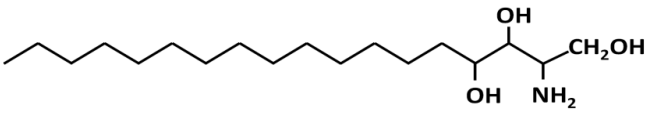
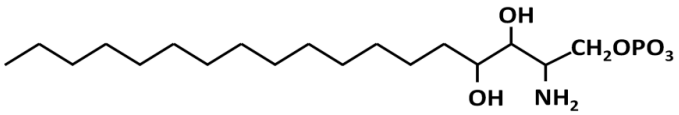
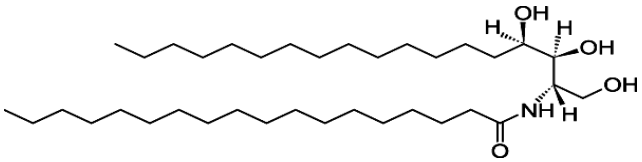
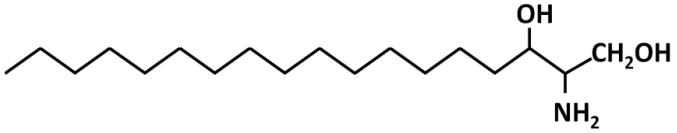
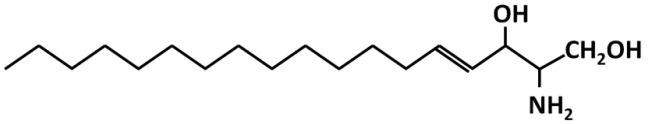
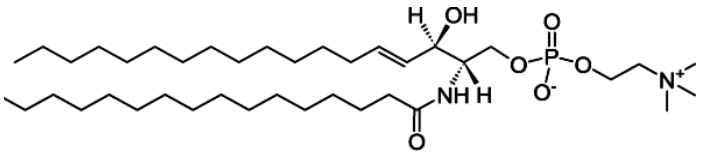
S. gordonii ATCC 35105 and *S. sanguinis* SK 36 were maintained on brain heart infusion (BHI) agar plates containing 37 g/l BHI (BD bioscience, USA) and agar (BD) under anaerobic conditions at 37 °C (Exterkate et al. 2010).

Preparation of sphingosines and coating of hydroxyapatite discs

Phytosphingosine (PHS, Doosan Corporation, South Korea) was a kind gift of Dr. P. Ekhart (Innopact BV, Ouderkerk aan de Amstel, the Netherlands). Sphingosine (egg,chicken), sphinganine(C-20), phytosphingosine-1-phosphate (PHS-1-PO_4, *Saccharomyces cerevisiae*), stearoyl phytosphingosine (stearoyl-PHS, *S. cerevisiae*) and sphingomyelin (egg, chicken) were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA) (Table 1). The HA discs (diameter 9.5 mm, thickness 2 mm, sintered at 1200 °C for 150 min) were obtained from Himed (NY, USA).

The stock solution of sphingomyelin was prepared in absolute ethanol at a concentration of 25 mg/ml. For the other sphingosines the concentrations of the stock solutions were 5 mg/ml. The working concentrations of the sphingosines were prepared in 20 mM Tris-HCl buffer (pH 6.8) supplemented with 0.1% Tween-20 to prevent aggregation of sphingosines. The HA discs were treated with 1.5 ml of sphingosines at a concentration of 100 µg/ml with gentle shaking (60 rpm) for 18 h at 37 °C. Subsequently the discs were washed 3 times with Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.5) supplemented with 1mM $CaCl_2$.

Table 1. Chemical structures of the sphingosines investigated in the present study.

Phytosphingosine (PHS)	
Phytosphingosine-1-phosphate (PHS-1-PO_4)	
Stearoyl phytosphingosine	
Sphinganine	
Sphingosine	
Sphingomyelin	

Bacterial adherence assay

Adherence of bacteria to HA discs was investigated using the active attachment model (Exterkate et al. 2010). This model consists of a custom made stainless steel lid with 24 clamps that contained the untreated and sphingosine-treated HA discs as substratum for adhesion of bacteria. Sphingosine-treated HA discs were washed 3 times by transferring the lid to a 24 well plate containing 1.6 ml of TBS and moved 10 times up and down to remove unbound sphingosines. Cultures of *S. gordonii* and *S. sanguinis* were grown anaerobically in BHI medium overnight at 37 °C and washed twice in TBS supplemented with 1mM $CaCl_2$. The final densities

of the cultures were adjusted to approximately 10^7 cells/ml. The untreated and sphingosine-treated HA discs were incubated anaerobically with 1.5 ml of bacterial suspension for 2 h at 37 °C and subsequently washed twice with TBS to remove the non-adherent bacteria. Then the discs with adherent bacteria were transferred to vials containing 2 ml TBS and the attached layer of bacteria was dispersed by sonication for 1 min with 1 sec pulses (Vibra cell, Newtown, USA). The resulting suspension was plated in different dilutions on BHI agar plates and incubated anaerobically for 48 h at 37 °C before CFUs were counted. The experimental group consisted of 3 HA discs and the experiment was performed twice.

Growth of biofilms

HA discs with adherent bacteria were prepared as described above. The discs were washed twice with cysteine peptone water (CPW, yeast extract 5 g/l, peptone 1 g/l, NaCl 8.5 g/l, L-Cysteine HCl 0.5 g/l) to remove non-adherent bacteria and subsequently the discs were incubated anaerobically in half-strength BHI medium (18.5 g/l BHI , 50 mM PIPES, pH 7) with or without 0.2% sucrose for 8 h at 37 °C. After 8 h of incubation, the medium was refreshed and the biofilm was grown for another 16 h under the same conditions to develop a 24 h old biofilm. The biofilms formed on the HA discs were washed twice with CPW to remove loosely adhered cells and transferred to vials containing 2 ml of CPW. The biofilms were dispersed by sonication and different dilutions were plated on BHI agar plates, incubated for 48 h and CFU counts was determined. The experiment was performed twice and in both cases each experimental group consisted of 6 HA discs.

Quantitative analysis of sphingosines adsorbed to HA discs

HA discs were incubated with 1.5 ml of sphinganine at concentrations ranging from 0 – 100 µg/ml with gentle shaking for 18 h at 37 °C. Similarly relative adsorption of PHS, sphingosine and sphinganine to HA discs were also evaluated at a concentration of 100 µg/ml. The discs were washed 3 times with demineralized (DM) water to remove unbound sphingosines. Discs were incubated twice with 1 ml of absolute ethanol with gentle shaking for 10 min to extract bound sphingosine. Controls incubations without HA discs were performed in parallel.

Extracts were pooled and evaporated. The residue was dissolved in 250 µl of absolute ethanol. To 100 µl of this solution 25µl *ortho*-phtaldialdehyde reagent (OPA, Merck, Darmstad, Germany, 500 mg/l OPA dissolved in 0.1 M tetraborate) was added to enable fluorimetric quantification of sphingosine. Fluorescence was measured with a fluorescense microtiter plate reader (Fluostar

Galaxy, BMG Laboratories, Offenburg, Germany) at 380 nm excitation and 450 nm emission wavelengths. The values of the controls (sphingosine adsorbed to wells) were subtracted from the values of the HA discs incubated at the same sphingosine concentration. The absolute quantities of sphingosines were determined by reference to the standard curves of sphingosines (concentrations from 0 – 100 µg/ml). All incubations were conducted in triplicate and the experiment was performed twice.

Confocal laser scanning microscopy of adherent bacteria

Sphinganine-treated HA discs were prepared by overnight incubation as described above. The sphinganine-treated discs and untreated control discs were incubated with cultures of *S. gordonii* and *S. sanguinis*, containing approximately 10^7 cells/ml, for 2 h and washed twice with sterilized DM water to remove non-adherent bacteria. Subsequently, the discs were fixed with 1.5 ml of 2.5% of glutaraldehyde for 2 h at 4 °C. The discs were washed 3 times with 1 ml of DM water to remove excess glutaraldehyde. Each disc was incubated with 750 µl of 60 µM propidium iodide (PI) solution (Invitrogen, Oregon, USA) in the dark for 20 min at room temperature. Subsequently the discs were washed 3 times to remove excess PI.

Confocal imaging was carried out with a fixed-stage TCS SP2 confocal microscope (Leica microsystems, Milton Keynes, UK) using an Ar laser with 490 nm excitation and 635 nm emission wavelengths for PI. The objective lens used was x40 water-immersion lens (Leica Microsystems) and the images were digitally magnified x4.

Hydrophobicity test

Hydrophobicity of bacteria was determined essentially as described previously (Weerkamp et al. 1988). In short, overnight grown cultures of *S. gordonii* and *S. sanguinis* were washed and suspended in TBS to a final optical density of 1.0. 1.2 ml of the cell suspension was mixed with 75 µl of hexadecane for 60s by use of a vortex mixer. The hexadecane layer was allowed to settle for 5 min and the optical density of the aqueous layer was determined. The percentage of bacteria in aqueous layer was calculated from the optical densities of the suspension at 600nm, before and after being mixed with hexadecane.

Statistical analysis

The statistical analysis was performed using the statistical software package IBM SPSS version 20 (IBM, Armonk, NY, USA). The data for the adherence assay and the biofilm assay of *S.*

gordonii and *S. sanguinis* to sphingosine-treated HA discs were analyzed using one-way ANOVA, followed by LSD tests as post-hoc procedures to determine significance differences between experimental groups. P values < 0.05 were considered statistically significant.

Results

Effect of sphingosine coatings on bacterial adhesion

The anti-adherent properties of a number of sphingosines were evaluated. The untreated and sphingosine-treated HA discs were exposed to suspensions of *S. gordonii* and *S. sanguinis* in TBS for 2 h. Subsequently the number of adhered bacteria was determined by culturing. Pretreatment of HA discs with sphinganine or PHS-1-PO_4 resulted in approximately 80 fold decrease in adherence of *S. gordonii*. Treatment with stearoyl-PHS decreased adherence by 5-fold, whereas sphingosine, PHS and sphingomyelin coatings had no significant effect on the adherence of *S. gordonii* (Fig. 1A). Sphinganine coatings inhibited the adherence of *S. sanguinis* also drastically (approximately 100-fold). Coating of HA with PHS-1-PO_4 had a smaller inhibitory effect on the adherence of *S. sanguinis* (5-fold) than on the adherence of *S. gordonii* (80-fold) (Fig. 1B). In contrast, PHS had virtually no effect on the adherence of *S. gordonii* and *S. sanguinis*. Coating of HA with stearoyl-PHS inhibited the adherence of *S. sanguinis* to the same extent as that of *S. gordonii* by 5-fold. To get insight in the cause underlying the different sensitivity of these two bacterial species to the various sphingosine coatings, the relative hydrophobicities were characterized by determining their adherence to hexadecane. This revealed that only 23% of *S. gordonii* was present in the hexadecane layer versus 52% of *S. sanguinis*, indicating that *S. sanguinis* is substantially more hydrophobic than *S. gordonii*.

To exclude that the low anti-adherence properties of PHS were caused by a diminished coverage of the HA surface, the amounts of PHS, sphingosine and sphinganine adsorbed to the HA discs were determined. This revealed that approximately 11 μg PHS bound per HA disc, which was somewhat higher than the amounts of adsorbed sphingosine and sphinganine (8 and 5 μg respectively). Because sphinganine showed the highest and most consistent anti-adherence effects on both *S. gordonii* and *S. sanguinis*, this compound was studied in more detail.

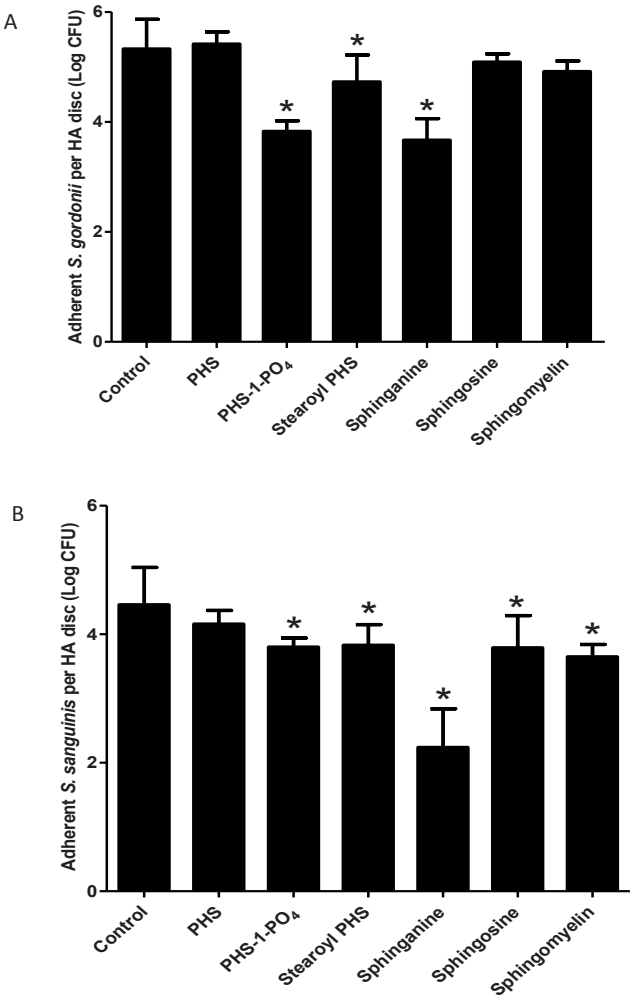


Figure 1. Effect of treatment of HA discs with different sphingosines on the adherence of *S. gordonii* (A) and *S. sanguinis* (B). The number of adherent bacterial cells per HA disc is expressed as log CFU. N=6, *P < 0.05 vs control.

Concentration dependent anti-adherence effect of sphinganine

To examine the concentration dependency of the anti-adherence effect of sphinganine, the HA discs were incubated with different concentrations of sphinganine ranging from 0-100 µg/ml. The amount of bound sphinganine increased over the concentration range studied (Fig. 2A), with maximal binding occurring when discs had been coated with 100 µg/ml sphinganine. Pretreatment of HA with 50 µg/ml sphinganine caused an approximately 10-fold decrease in adherence of *S. gordonii* and *S. sanguinis*. The decrease in adherence was the highest (approximately 100-fold) at a sphinganine concentration of 100 µg/ml (Fig. 2B).

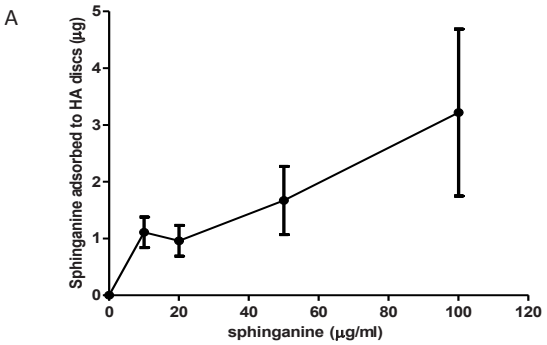


Figure 2A. The amount of sphinganine adsorbed to HA discs after treatment with different concentrations of sphinganine (0-100 µg/ml). Data are expressed as µg of sphinganine adsorbed per HA disc (mean ± SD, N=3).

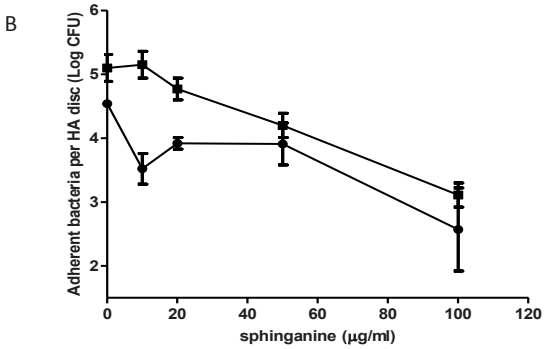


Figure 2B. Adherence of *S. gordonii* (squares) and *S. sanguinis* (dots) on sphinganine-treated HA discs treated with different concentrations of sphinganine (0-100 µg/ml). The number of adherent bacterial cells per HA disc is expressed as log CFU (mean ± SD, N=3).

Confocal laser scanning microscopy of adherent bacteria

The decrease in adherence of bacteria to sphinganine-treated HA discs was further explored by confocal microscopy (Fig. 3). This revealed that *S. gordonii* adhered to the surface of the HA disc as short chains, while *S. sanguinis* was present as long chains. On sphinganine-treated HA a clear decrease in the number of adhered *S. gordonii* and *S. sanguinis* was observed, supporting that the observed effects were primarily caused by a decreased binding of bacteria, rather than by a direct bactericidal effect of sphinganine.

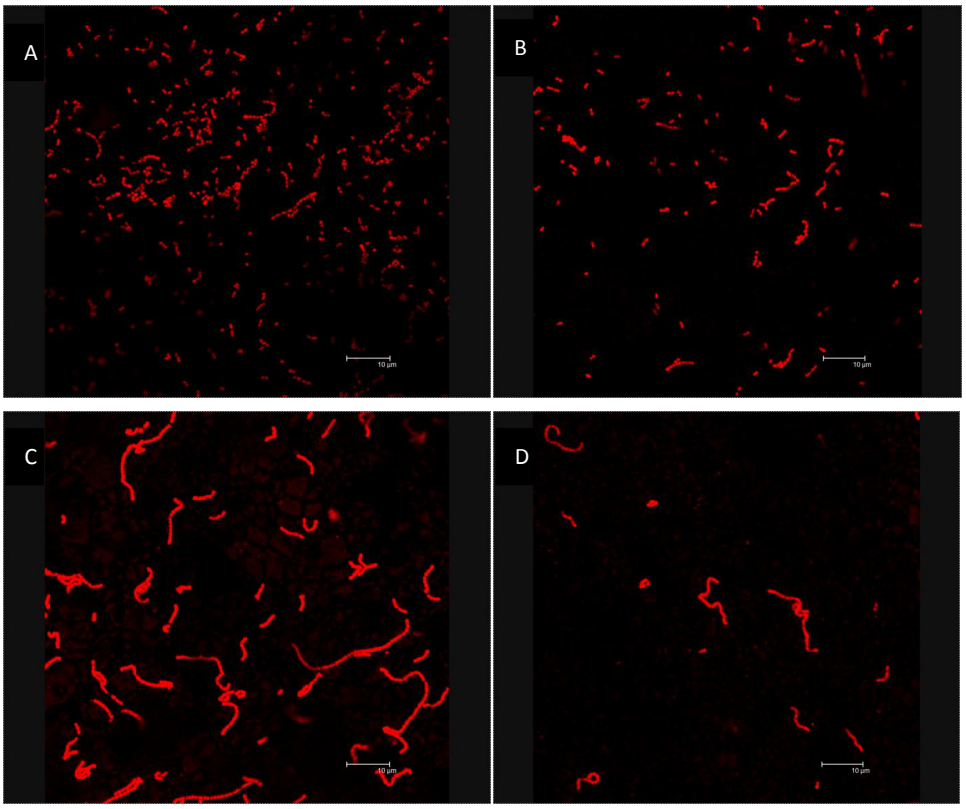
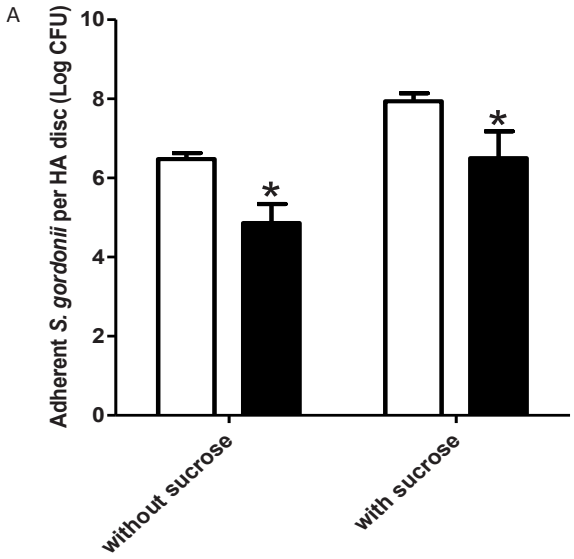


Figure 3. Confocal micrographs showing *S. gordonii* adherent to untreated (A) and sphinganine-treated (B) HA discs. Similarly, micrographs of *S. sanguinis* to untreated (C) and sphinganine-treated (D) HA discs are shown. The HA discs were treated with sphinganine at a concentration of 100 µg/ml. Scale bar represents 10 µm.

Effect of sphinganine coating on biofilm formation

In the previous experiments, the initial adherence (2 h incubation) of bacteria was examined in TBS, a condition which prevents growth of bacteria. To evaluate the effect of sphinganine coating under conditions that support growth of bacteria, sphinganine coated discs were exposed for 24 h to bacteria in growth medium, with and without 0.2% sucrose. Both *S. gordonii* and *S. sanguinis* are capable of producing dextran from sucrose, which is important for biofilm formation. Under growth supporting conditions, *S. gordonii* exhibited a significant 75-fold decrease in growth, both in the presence and in the absence of sucrose (Fig. 4A).With *S. sanguinis*, a significant 100-fold decrease in growth was observed only in the absence of sucrose. When discs were exposed to *S. sanguinis* in the presence of sucrose, inhibition of the *S. sanguinis* biofilm was no longer observed (Fig. 4B).



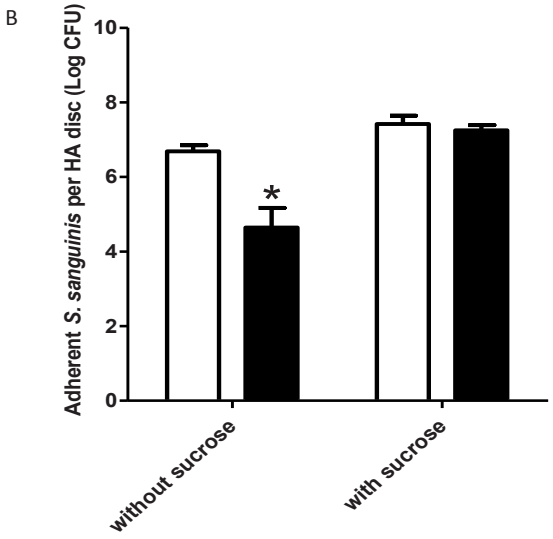


Figure 4. Development of a 24 h old biofilm of *S. gordonii* (A) and *S. sanguinis* (B) on untreated (white bars) and sphinganine-treated (black bars) HA discs. The number of adherent bacterial cells per HA disc is expressed as log CFU. N=6, *P < 0.05 vs untreated HA discs cultured under the similar conditions.

Discussion

Bacterial adherence to surfaces is governed by several factors such as the physicochemical properties of the surface, cell wall properties of bacteria and the composition of the liquid medium. Physicochemical properties of the surface include short range forces like donor-acceptor, hydrogen, ionic, covalent and coordinate bonds, and long range forces such as electrostatic and van der Waals forces or stereochemical molecular recognition interactions (Rouxhet and Mozes 1990; Katsikogianni and Missirlis 2004; Renner and Weibel 2011).The present study showed that members of the sphingosine family formed stable films on HA, which effectively inhibit adherence of *S. gordonii* and *S. sanguinis*. Previously it has been shown that sphingosines in solution exert killing effects on both Gram-positive and Gram-negative bacteria (Bibel et al. 1992; Bibel et al. 1993; Fischer et al. 2012). The present study indicates that members of the sphingosines family, bound to a HA surface, also effectively inhibit bacterial adhesion. The anti-adherence effects were most likely caused by the altered physicochemical properties of the HA surface, such as change in net charge and hydrophobicity, which play an important role in the primary adhesion of bacteria to the HA surface.

Despite a large similarity in structure, the anti-adherence properties of the various sphingosines differed widely. For instance sphinganine inhibited adherence of *S. gordonii* by 80-fold, whereas its unsaturated analogue sphingosine and its hydroxylated analogue PHS had no measurable effects (Fig. 1a). These three compounds share the same amino head groups and bind approximately to the same extent to HA, ruling this out as an explanation for the observed large differences in the anti-adherence effects. Although the molecular architecture of the sphingosine layer on HA still must be elucidated, on theoretical grounds it may be assumed that adsorbed on the HA surface, these amphoteric compounds will form aggregates (bilayers or micelle like) with the positively charged head groups exposed to the bulk fluid, as well as adhered onto the HA surface. Sphinganine having a saturated acyl chain will likely form a more rigid film than its unsaturated or hydroxylated analogues. Therefore we envisage that sphinganine films are less vulnerable to disruption e.g. by bacterial adhesins than the more fluid films composed of sphingosine or PHS.

Interestingly, though *S. gordonii* and *S. sanguinis* belong to the same mitis taxonomic group, they were both maximally inhibited by sphinganine, but were affected differently by the other sphingosines such as sphingosine, PHS-1-PO_4 and sphingomyelin (Fig. 1a and 1b). This variation may be attributed to differences in the hydrophobicity of the two bacterial strains, with *S. sanguinis* (52% in hexadecane layer) comparatively more hydrophobic than *S. gordonii* (23% in

hexadecane layer). *S. gordonii* was inhibited by sphinganine, PHS-1-PO_4 and stearoyl PHS. In addition, adherence of *S. sanguinis* was also inhibited by sphingosine and sphingomyelin. Presumably, the charged head groups of sphingosines will affect the hydrophobic *S. sanguinis* more than the comparatively hydrophilic *S. gordonii*. On the other hand PHS-1-PO_4, which is even more hydrophilic, inhibited *S. gordonii* to the same extent as sphinganine. This may be due to the repulsive forces between the phosphate group of sphingosine and the negatively charged groups on the bacterial surface.

Biofilms were grown in the presence and absence of sucrose to investigate whether extracellular polysaccharides, synthesized in the presence of sucrose, will affect the inhibitory effect of sphinganine. Growth of *S. sanguinis* on sphinganine-treated HA discs was reduced by 100-fold in absence of sucrose but in presence of sucrose the inhibitory effect was lost (Fig 4b). We speculate that in the absence of sucrose *S. sanguinis*, which is hydrophobic, lacks chemical groups in the cell surface which example via hydrogen bonds can associate with the hydrophilic head groups of a sphinganine coating. In the presence of sucrose, however, hydrophilic extracellular polysaccharide is synthesized which can give rise to hydrogen bonding, enabling a larger number of bacteria to bind. The first layer of bacteria will then function as a substratum on which the biofilm can develop.

A limitation of the present study may be that the effects of sphingosines on the adherence of bacteria were studied with monocultures of bacteria. Therefore, subsequent studies are needed to explore the possible effects of sphingosines on the growth and composition of mixed biofilms. Despite this limitation, the present study suggests that sphingosines, in particular sphinganine, have potential as a coating material for preventing adherence and growth of bacteria on biomaterials.

Acknowledgement

We thank Mr. Cor Semeins, Mr. Ton Schoenmaker and Dr. Bastiaan Krom for their help with confocal microscopy. We are also thankful to Mr. Rob Exterkate for his help with the active attachment model; Mr. Michel Hoogenkamp and Dr. Dongmei Deng for providing the bacterial cultures. Financial support from Stichting Toegepaste Wetenschappen (STW), grant 10184 is gratefully acknowledged.

References

Baehni PC, Takeuchi Y. 2003. Anti-plaque agents in the prevention of biofilm-associated oral diseases. *Oral Dis*. 9:23-29.

Bibel DJ, Aly R, Shinefield HR. 1992. Antimicrobial activity of sphingosines. *J Invest Dermatol*. 98:269-273.

Bibel DJ, Aly R, Shah S, Shinefield HR. 1993. Sphingosines: antimicrobial barriers of the skin. *Acta Derm Venereol*. 73:407-411.

Bikker FJ, Cukkemane N, Nazmi K, Veerman ECI. 2013. Identification of the hydroxyapatite binding domain of salivary agglutinin. *Eur J Oral Sci*. 121:7-12.

Corbin A, Pitts B, Parker A, Stewart PS. 2011. Antimicrobial penetration and efficacy in an *in vitro* oral biofilm model. *Antimicrob Agents Chemother*. 55:3338-3344.

Domon-Tawaraya H, Nakajo K, Washio J, Ashizawa T, Ichino T, Sugawara H, Fukumoto S, Takahashi N. 2013. Divalent cations enhance fluoride binding to *Streptococcus mutans* and *Streptococcus sanguinis* cells and subsequently inhibit bacterial acid production. *Caries Res*. 47:141-149.

Egland PG, Du LD, Kolenbrander PE. 2001. Identification of independent *Streptococcus gordonii* sspA and sspB functions in coaggregation with *Actinomyces naeslundii*. *Infect Immun*. 69:7512-7516.

Fischer CL, Drake DR, Dawson DV, Blanchette DR, Brogden KA, Wertz PW. 2012. Antimicrobial activity of sphingoid bases and fatty acids against Gram-positive and Gram-negative bacteria. *Antimicrob Agents Chemother*. 56:1157-1161.

Gibbons RJ, Hay DI, Schlesinger DH. 1991. Delineation of a segment of adsorbed salivary acidic proline-rich proteins which promotes adhesion of *Streptococcus gordonii* to apatitic surfaces. *Infect Immun*. 59:2948-2954.

Gong K, Mailloux L, Hezberg MC. 2000. Salivary film expresses a complex, macromolecular binding site for *Streptococcus sanguis*. *J Biol Chem*. 275:8970-8974.

Haffajee AD, Socransky SS**.** 1994. Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000*. 5:78–111.

Jenkinson HF, Lala HC, Shepherd MG. 1990. Coaggregation of *Streptococcus sanguinis* and other streptococci with *Candida albicans*. *Infect Immun*. 58:1429-1436.

Katsikogianni M, Missirlis YF. 2004. Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions. *Eur Cell Mater*. 8:37-57.

Kolenbrander PE, Andersen RN, Blehert DS, Egland PG, Foster JS, Palmer RJ Jr. 2002. Communication among oral bacteria. *Microbiol Mol Biol Rev*. 66:486-505.

Kuboniwa M, Tribble GD, James CE, Kilic AO, Tao L, Hezberg MC, Shizukuishi S, Lamont RJ. 2006. *Streptococcus gordonii* utilizes several distinct gene functions to recruit *Porphyromonas gingivalis* into a mixed community. *Mol Microbiol*. 60:121-139.

Lamont RJ, El-Sabaeny A, Park Y, Cook GS, Costerton JW, Demuth DR. 2002. Role of the *Streptococcus gordonii* sspb protein in the development of *Porphyromonas gingivalis* biofilms on streptococcal substrates. *Microbiology*. 148:1627-36.

Ofek I, Hasty DL, Sharon N. 2003. Anti-adhesion therapy of bacterial diseases: prospects and problems. *FEMS Immun Med Micro*. 28:181-191.

Oliveira MRTR, Napimoga MH, Cogo K, Goncalves RB, Macedo MLR, Freire MGM, Groppo FC. 2007. Inhibition of bacterial adherence to saliva-coated through plant lectins. *J Oral Sci*. 49:141-145.

Ooshima T, Minami T, Aono W, Izumitani A, Sobue S, Fujiwara T, Kawabata S, Hamada S. 1993. Oolong tea polyphenols inhibit experimental dental caries in SPF rats infected with mutans streptococci. *Caries Res*. 27:124-129.

O'Sullivan JM, Jenkinson HF, Cannon RD. 2000. Adhesion of *Candida albicans* to oral streptococci is promoted by selective adsorption of salivary proteins to the streptococcal cell surface. *Microbiology*. 146:41-48.

Renner LD, Weibel DB. 2011. Physicochemical regulation of biofilm formation. *MRS Bull*. 36:347-355.

Rogers JD, Palmer RJ, Kolenbrander PE, Scannapieco FA. 2001. Role of *Streptococcus gordonii* amylase-binding protein A in adhesion to hydroxyapatite, starch metabolism, and biofilm formation. *Infect Immun*. 69:7046-7056.

Rosan B, Lamont RJ. 2000. Dental plaque formation. *Microbes Infect*. 2:1599-1607.

Rouxhet PG, Mozes N. 1990. Physical chemistry of the interface between attached micro-organisms and their support. *Water Sci Technol*. 22:1-16.

Sansone C, Van Houte J, Joshipura K, Kent R, Margolis HC. 1993. The association of mutans streptococci and non-mutans streptococci capable of acidogenesis at a low pH with dental caries on enamel and root surface. *J Dent Res*. 72:508-516.

Shimotoyodome A, Koudate T, Kobayashi H, Nakamura J, Tokimitsu I, Hase T, Inoue T, Matsukubo T, Takaesu Y. 2007. Reduction of Streptococcus mutans adherence and dental

biofilm formation by surface treatment with phosphorylated polyethylene glycol. Antimicrob Agents Chemother. 51:3634-3641.

Weerkamp AH, Uyen HM, Busscher HJ. 1988. Effect of zeta potential and surface energy on bacterial adhesion to uncoated and saliva-coated human enamel and dentin. *J Dent Res*. 67:1483-1487.